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IMPROVING ENDOMETRIAL CARCINOMA DIAGNOSTICS

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Improving endometrial carcinoma diagnostics

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Endometrial carcinoma is the most common gynecological malignancy and greatly affects global disease burden. Despite it being among the most common cancers it is relatively understudied and until recently poorly understood. Diagnostics of endometrial carcinoma have been underdeveloped for many years resulting in perpetual poor interobserver reproducibility in turn inhibiting correct diagnosis and complicating comparisons of clinical trials. In this thesis we used a three-tiered approach in trying to improve endometrial carcinoma diagnostics: strictly improving morphological criteria, adding immunohistochemistry (IHC)/biomarker panels, as well as genomics data in order to improve diagnostic and prognostic ability.

In **study 1** we compared standard FIGO grading of endometrial carcinoma to a newly developed morphologic system we named cell-type independent (CTI). We compared interobserver reproducibility using both systems in 70 patient`s endometrial biopsies. We could not identify any significant improvement in the form of increased interobserver reproducibility nor increased accuracy when compared to final hysterectomy diagnosis. We conclude that the limitations of the biopsy setting hampered the CTI system`s ability to outperform current FIGO grading system and that the assessment of nuclear grade, no matter what the definitions of a high nuclear grade are, remain subjective.

In **study 2** 12 separate subspecialized gynaecological pathologists reviewed pathology slides of 70 cases of endometrial biopsies. We compared the interobserver agreement when adding a biomarker panel (IHC for p53, ER and PGR and DNA ploidy analysis) to only standard morphology diagnosis with accompanying results of final hysterectomy diagnosis. The addition of a biomarker panel increased interobserver agreement from 75,8%, kappa = 0.52 to 84%, kappa= 0.68. Diagnostic agreement to final hysterectomy diagnosis also increased with use of the panel, going from 83.6% to 88.7% agreement with final diagnosis after incorporating the panel in biopsy diagnosis ($p<0.05$). The two biomarkers p53 IHC and DNA ploidy analysis showed significant effect on up-or downgrading of tumors. We conclude that selective use of a simple biomarker panel greatly aids in endometrial biopsy diagnostics.

Study 3 was a collaborative effort between Bern and Karolinska University Hospital we sought to implement a surrogate molecular marker in 604 patients with endometrial carcinoma in order to try to replicate the TCGA (The cancer genome atlas) molecular markers classifiers and to hopefully aid in endometrial carcinoma diagnostics and prognostics. The panel consisted of IHC for p53 (to define a p53mut group) and MSH2, MSH6, PMS2, MLH1 (to define a MMRd, mismatch repair defective, group) and Sanger sequencing of the POLE gene (to define a POLEmut) group. We found that this simple surrogate molecular marker, using readily available methods, could reproduce the findings of the TCGA and render four prognostically different molecular categories. We could however not prove an added prognostic benefit over current risk assessment prognostic

tools, nor any benefit in adding a surrogate molecular marker to current prognostic markers. Moreover only one of the molecular groups (p53 mut) proved to be statistically significant marker for predicting Progression free survival (PFS) and overall survival (OS). From this we concluded that in the setting of non-selected group of patients consisting mostly of low grade tumors proving significant differences between each of the molecular groups couldn't be done since progression events were so rare and the group as a whole behaved indolently. Nonetheless there are clear benefits with the addition of genomic data for other purposes such as aid in suitable patient selection for adjuvant treatment.

In **study 4** we conducted sequencing of exons 9-14 of the POLE gene in a cohort of 604 patients with endometrial carcinoma in order to more deeply analyze mutation events and the phenotype of POLE mutated endometrial carcinomas. We found mostly previously described hotspot mutations but also a substantial number of mutations with unknown significance and characterized POLEmut tumors as occurring in mainly younger women with nulliparity and consisting predominantly of endometrioid endometrial carcinomas. This study shows that when POLE mutations are clearly defined as either hotspot mutations and/or POLE mutations with a known hypermutated phenotype this patient group of POLEmut had very good outcomes with only 1 recurrence in 38 patients. The Cox regression couldn't however show any significant difference between POLEmut and non POLEmut tumors, however we believe again this was because we are dealing with a low risk EC population where both recurrence events and POLE mutations are rare, and thus we believe we were underpowered.

LIST OF SCIENTIFIC PAPERS

I. **Nastic D**, Kahlin F, Dahlstrand H, Carlson JW. A Cell Type Independent Binary Grading System Does Not Significantly Improve Endometrial Biopsy Interpretation. Int J Gynecol Pathol. 2016 May;35(3):256-63

II. **Nastic D**, Shanwell E, Wallin KL, Valla M, Måsbäck A, Mateoiu C, Lidang M, Liakka A, Lappi-Blanco E, Grove A, Davidson B, Carpen O, Bertelsen BI, Bak J, Abusland AB, Selling J, Carlson JW. A Selective Biomarker Panel Increases the Reproducibility and the Accuracy in Endometrial Biopsy Diagnosis. Int J Gynecol Pathol. 2017 Jul;36(4):339-347.

III. Sara Imboden,* **Denis Nastic**,* (contributed equally) Mehran Ghaderi, Filippa Rydberg, Daniel Olsson, Michael Mueller, Tilman Rau, Elisabeth Epstein, Joseph W. Carlson. Molecular classification of endometrial cancer provides complementary information, but does not outperform current predictive models: the Karolinska and Bern experience. Manuscript.

IV. Imboden S, **Nastic D** (contributed equally), Ghaderi M, Rydberg F, Rau TT, Mueller MD, Epstein E, Carlson JW. Phenotype of POLE-mutated endometrial cancer. PLoS One. 2019 Mar 27;14(3):e0214318.

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I. Eriksson LSE, **Nastic D**, Frühauf F, Fischerova D, Nemejcova K, Bono F, Franchi D, Fruscio R, Ghioni M, Haak LA, Hejda V, Meskauskas R, Opolskiene G, Pascual MA, Testa A, Tresserra F, Zannoni GF, Carlson JW, Epstein E. Clinical and Ultrasound Characteristics of the Microcystic Elongated and Fragmented (MELF) Pattern in Endometrial Cancer According to the International Endometrial Tumor Analysis (IETA) criteria. Int J Gynecol Cancer. 2019 Jan;29(1):119-125.

II. Carlson JW, **Nastic D**. High-Grade Endometrial Carcinomas: Classification with Molecular Insights. Surg Pathol Clin. 2019 Jun;12(2):343-362.

III. Eriksson LSE, **Nastic D**, Lindqvist PG, Imboden S, Järnbert,Pettersson H, Carlson JW, Epstein E. Sonographic, demographic characteristics, and the Proactive Molecular Risk Classifier for Endometrial cancer (ProMisE) in the prediction of tumor recurrence or progression. Ultrasound Obstet Gynecol. 2020 Dec 14. Epub ahead of print.

CONTENTS

LIST OF ABBREVIATIONS

CN	Copy number
CRC	Colorectal cancer
CT	Computed tomography
CTRT	Combination of chemo-and radiotherapy
D&C	Dilatation and curettage
DOI	Depth of invasion
EBRT	External beam radiation therapy
EC	Endometrial carcinoma
EIN	Endometrial intraepithelial neoplasia
ER	Estrogen receptor
ESGO	European Society of Gynaecological Oncology
ESMO	European Society for Medical Oncology
ESTRO	European Society for Radiotherapy & Oncology
GOG	Gynecologic oncology group
HNF1B	Hepatocyte nuclear factor 1 homeobox B
IHC	Immunohistochemistry
LA	Lymphadenectomy
LVSI	Lymphovascular invasion
MMRd	Mismatch repair deficient
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
NCCN	National comprehensive cancer network
NGS	Next-generation sequencing

P53mut	P53 mutated
P53wt	P53 wild-type
PCR	Polymerase chain reaction
PFS	Progression free survival
POLE	Gene coding the catalytic subunit of DNA polymerase epsilon
PORTEC	Post-operative radiation therapy of endometrial carcinoma
PR	Progesterone receptor
PRG	Progesterone receptor
ProMisE	Proactive Molecular Risk Classifier for Endometrial Cancer
RT	Radiotherapy
SHBG	Sex hormone binding globulin
TFD	Tumor free distance
TILs	Tumor infiltrating lymphocytes
TMB	Tumor mutational burden
TVA	Transvaginal ultrasound
VBT	Vaginal brachytherapy

1 INTRODUCTION

Endometrial carcinoma (EC) in a lot of ways represents the female counterpart of prostate cancer. It is a common cancer although not widely known, it affects mostly older women, and the outcomes are generally good with relatively few cancers causing death from disease. Frequently, patients experience symptoms early in the cancer genesis and thus are discovered early, have no spread beyond primary site and are often cured with surgery alone. Similar to prostate cancer the EC research area is largely underdeveloped in comparison to more research-intensive cancers such as breast or lung. This has in part, as will be described below, led to a poor understanding of EC tumor biology, ambiguous histopathological diagnostics and non-standardized and sometimes somewhat arbitrary treatment strategies.

1.1 EPIDEMIOLOGY AND ETIOLOGY

EC is the most common gynecological cancer, outnumbering all the other gynecological cancers combined. It is the sixth most common cancer in Swedish women with 1294 reported cases in 2018[1]. In 2018 roughly 382000 new cases were reported and 90000 deaths attributable to EC[2,3] worldwide, making it the sixth most common cancer in women worldwide. Survival and mortality rates (5-year survival rates are roughly 80% for the whole patient group, and mortality rates 4/100000 women[4]) are relatively good, mostly due to EC causing early symptoms and thus being detected in early stages[5]. While the incidence rate in Sweden has been steady for the last decade it is believed the rates will rise in the future as the trend in most of the western world is a slow and steady increase in incidence rates[6]. Incidence is roughly 4-5 times higher in the western compared to the developing world. The increasing incidence and the geographical differences are attributed to lifestyle-factors such as increasing obesity, diabetes mellitus and an aging population. As these trends in developed countries continue, EC mortality, morbidity and global disease burden are expected to rise[3,7–9].

EC, in general, is caused by either of two etiologically distinct pathways (type 1 and 2 ECs), which differ in both histology and tumor genomics[10](see below). The first is the hyper-estrogenic pathway accounting for roughly 80% of EC cases (“type 1 EC”). In summary any condition or medication (exo- or endogenous) which disrupts the balance of progesterone and estrogen resulting in increasing or unopposed levels of estrogen causes hyperplasia of the endometrium thus increasing risk of cancer development[11,12].

The most common risk factor for hypoestrogenism is obesity. Fatty tissue aromatizes androstenedione to estrogen causing hyperestrogenism, which in the setting of obesity is unopposed by progesterone[13–15]. Infertility and nulliparity decreases levels of sex hormone binding globulin (SHBG) leading to increased levels of circulating estrogen[16]. Non-combination (without combination of progestins) Estrogen replacement treatment (usually

for menopausal symptoms) leads to a 6-fold increased risk of EC development[17], however only local estrogen treatment is not associated with an increased risk[18]. Tamoxifen, an ER blocking agent used to treat women with ER+ breast cancers, paradoxically increases risk of EC by activating a large number of cell proliferation mechanisms in the uterine lining[19,20]. Finally, polycystic ovarian syndrome (PCOS), can cause prolonged anovulatory periods leading to a persistent increase in circulating estrogen through a negative feedback-loop thus increasing risk of endometrial proliferation[21]. Patients following this first pathway tend to have tumors of endometrioid low-grade histology, low-stage and generally have better outcomes.

The second pathway ("Type 2 EC") accounts for 10-20% of EC cases. These cancers are not clearly associated with elevated estrogen levels, although their direct causal events are poorly understood. Progression to type 2 EC, seems to arise from an atrophic i.e. non-hyperestrogenic related background endometrium. There is a slight dichotomic age distribution in type 2 ECs, resulting in two age peaks, these patients tend to be older in comparison to type 1 ECs but at the same time type 2 ECs also occur in younger pre- and perimenopausal women to a much greater extent than type 1 ECs[22–24]. In general, these women tend to be of normal weight, older and multiparous. The histology of theses tumors tends to be high grade and non-endometrioid. Even though type 2 ECs account for a small minority of EC cases they are more aggressive, causing roughly 50% of EC mortality[25,26].

Hereditary EC accounts for 1-5% of EC cases. Patients with Lynch syndrome have mutations in DNA mismatch repair proteins (MSH2, MSH6, MLH1, PMS2) causing microsatellite instability (MSI) and tumors with high mutational burdens. Patients with Lynch syndrome run a 40-60% lifetime risk of EC development and are usually younger than 50 years at cancer debut[27,28]. Patients who debut at young age and have a family history suggestive of Lynch syndrome should be screened for Lynch syndrome either using immunohistochemistry (IHC) or via oncogenetic counselling[29–31]. If patients are found to have Lynch syndrome they should be monitored via regular gynecological examination and vaginal ultrasound, and after child bearing years are over recommended prophylactic hysterectomy and bilateral salpingoophrectomy[32]. Cowden syndrome is another form of hereditary EC, although much more rare than Lynch syndrome. Patients with Cowden syndrome have a germ-line mutation of the tumor-suppressor gene PTEN and a 1-5% lifetime risk of developing EC[33–35].

Smoking, grand multiparity (birth of more than 5 children), and contraceptive progestin pills reduce the risks of developing EC[17,36–38]. This is accomplished by reducing effects of estrogen either by inducing higher metabolism (smoking), or by counteracting the effects of estrogen (progestin contraceptive pills, intrauterine devices and multiparity).

1.2 SYMPTOMS AND INITIAL CLINICAL ASSESSMENT

The most common symptoms prompting clinical investigation for possible EC are postmenopausal bleeding, menorrhagia (excessive menstrual bleeding), pyometra and vaginal discharges[39]. Of note, a small number of ECs are incidental discoveries, either via imaging modalities done for other symptoms or after hysterectomies for other common causes (such as resections of leiomyoma).

The first step in the clinical investigation involves gynecological examination including a transvaginal ultrasound (TVA). TVA helps identify patients where an endometrial biopsy is necessary; if the endometrium thickness is over 5 mm an endometrial biopsy is recommended to rule out EC as cause of bleeding[39,40]. However, if clinical suspicion of EC is high one should proceed to biopsy immediately to reduce the risk of delayed diagnosis and therefore treatment delay.

Mandatory first steps when evaluating patients with EC include an examination of family and medical history that may suggest risk of a potential hereditary (Lynch syndrome) cause of cancer. Since many patients are older and comorbid a physical examination is needed to determine if any possible limitations of treatment exist, since not all patients will be able to endure the full extent of possible treatment even if it is deemed necessary. Once a diagnosis of EC has been made the next step is clinical and radiologic staging in order to help determine what treatment is best suited for the patient. The first steps include a TVA, pelvis sonography and/or Magnetic resonance imaging (MRI) to help determine myometrial depth invasion, tumor size, possible cervical invasion, lymph node metastasis, serosal involvement and/or possible local pelvic spread[41,42]. If histology shows a high risk/type 2 EC (more on that below) a computed tomography (CT) of the chest and pelvis can be performed as to rule out possible metastases, however it rarely affects treatment choice since very few patients have metastasized disease at debut (2-3%)[43].

1.3 HISTOPATHOLOGY

The WHO classification of EC has not changed much when comparing previous[44] to current versions[45], the histological classification has largely remained the same for a long time. It remains in line with the Bokhman[10], two-tiered EC classification. The histopathology classification is based on tumor *histotype* and *grade*. This assessment categorizes patients into high and low risk groups, as tumor grade is highly associated with prognosis[46]. Therefore, the initial biopsy diagnosis of a tumor has a large impact on the patient, as it affects extent of surgery (lympadenectomy or no lymphadenectomy), possible neoadjuvant treatment and extent of initial clinical workup (see more on this below in risk assessment).

The most important initial designation a pathologist makes is determining the tumor *histotype*, dividing tumors into endometrioid and non-endometrioid types. All non-

endometrioid tumor types are by definition classified as high grade, whereas endometrioid tumors are further graded according to FIGO system (see below) of endometrioid tumors[47].

Endometrioid ECs represent the most common type, accounting for roughly 80% of EC cases[25]. These tumors originate in a background of hyper-estrogenic hyperplastic proliferative endometrium often with endometrioid intraepithelial neoplasia (EIN)/atypical complex hyperplasia[48,49]. The morphology of endometrioid ECs is reminiscent of normal proliferating endometrium. The prototypical growth pattern is of complex and jagged anastomosing glands forming glandular back-to-back patterns with cribriform structures. The epithelial lining consists of atypical large cylindrical cells with central large nuclei and an eosinophilic cytoplasm. These tumors often show greatly heterogenous morphology, including large areas of squamous, mucinous, solid, villoglandular and secretory differentiation[50]. Because of these heterogenous features they can be challenging to diagnose, especially with limited biopsy material. For endometrioid ECs the second step is to assess the *histological grade,* this determines if the tumor is low- or high grade (only endometrioid tumors undergo this second-step of grading as non-endometrioid histotypes are immediately graded as high-grade). This is done using the FIGO/WHO grading system[45,47,51]:

1. Grade 1 tumors have <5% solid tumor growth.
2. Grade 2 tumors have between 5-50% solid tumor growth.
3. Grade 3 tumors have >50% solid tumor growth.

Note that when assessing the extent of solid growth one must disregard solid areas with squamous differentiation, which can be difficult to distinguish from solid growth glandular components[52,53]. In addition to assessing and grading the architecture/extent of solid growth of a tumor, the degree of nuclear atypia is also assessed. If a tumor shows high grade nuclear atypia, deemed inappropriately high for its architecture, the tumor grade is upgraded by one grade[54–57]. In other words, a tumor with 30% solid growth (grade 2 architecturally) but with high grade nuclear atypia is upgraded to a grade 3 tumor. Grade 1-2 tumors endometrioid ECs are deemed low grade/low risk while grade 3 are high grade tumors with a substantially increased risk of metastasis and recurrent disease[58–60].

Of the non-endometrioid i.e. high grade tumors **serous carcinomas** are the most common representing roughly 1-5% of all ECs[25]. They generally affect older women in a background of atrophic endometrium. This is the most aggressive type of EC often presenting with peritoneal and lymphatic spread, with deep myometrial invasion and with high risk of local and distant recurrance[26,61,62]. They are morphologically characterized by papillary and micropapillary fronds and outgrowths that are lined by tightly packed highly atypical tumor nuclei. Tumor necrosis and a high mitotic rate are common. The lumens of the glands and papillary structures show an uneven undulating surface, which can be a helpful diagnostic clue in trying to separate serous ECs from high grade endometrioid tumors.

Less common but nonetheless regularly encountered variant of non-endometrioid carcinoma is **clear cell carcinoma**, representing about 2% of all EC cases[25]. It is important to be aware of this entity as it behaves aggressively clinically, often show distant metastasis and has a high risk of extensive distant spread[26,63–65]. Morphologically these tumors grow in a solid, tubulocystic or villous pattern and are lined by the defining feature of large polygonal cells with clear or bright slightly eosinophilic cytoplasm. These lining cells usually form a single layer on the underlying stroma sometimes forming another classical clear cell carcinoma feature of "hobnailing", where the apical part of tumor cells protrude into the lumen imparting a hobnail appearance. The nucleic atypia varies from mild to focal severe and sometimes bizarre looking atypia. These cytologic features are important to notice as clear cell carcinoma architecturally can grow in a very similar pattern to serous carcinomas showing prominent papillary features[66,67].

The remaining high-grade EC variants are exceedingly rare but are worth mentioning as they behave highly aggressively clinically and are responsible for a substantial portion of cancer related deaths in EC. Firstly **carcinosarcoma** (previously called malignant mixed Mullerian tumor, MMT) is composed of both malignant epithelial and mesenchymal elements, essentially leading to mixed carcinoma and sarcoma appearance in the microscope. Both elements are high grade. Recent molecular and IHC studies have shown that carcinosarcomas aren't genuine mixed tumors but rather a sarcomatoid dedifferentiation of the epithelial component and the driver of the malignancy lies within the epithelial component[23,68,69], therefore carcinosarcoma is best regarded as a metaplastic carcinoma. Secondly, a recent addition, is the variant of EC called **dedifferentiated or undifferentiated** EC (same entity but the name changes depending on if there remains a low grade remnant from which the de-/undifferentiated component arose from). These tumors are important as they can be difficult to differentiate from a grade 3 endometrioid EC but behave even more aggressively. Morphologically these tumors grow in a solid patternless non glandular pattern and are composed of small tumor cells with prominent dyscohesion[70–72].

Finally, ECs don't just display growth pattern plasticity within each tumor category described above, they also show mixed histotype components. As many as 5% of ECs show features of mixed carcinoma[73]. **Mixed carcinomas** are composed of two or more distinctly clear histotypes, each one representing at least 5% of the tumor. The most common mixed carcinomas are mixed serous and endometrioid followed by mixed clear cell and endometrioid. These tumors are important to diagnose correctly as it seems they clinically behave as their worst component, that is a mixed carcinoma with a small serous component still tends to behave as a "pure" serous carcinoma[74–76].

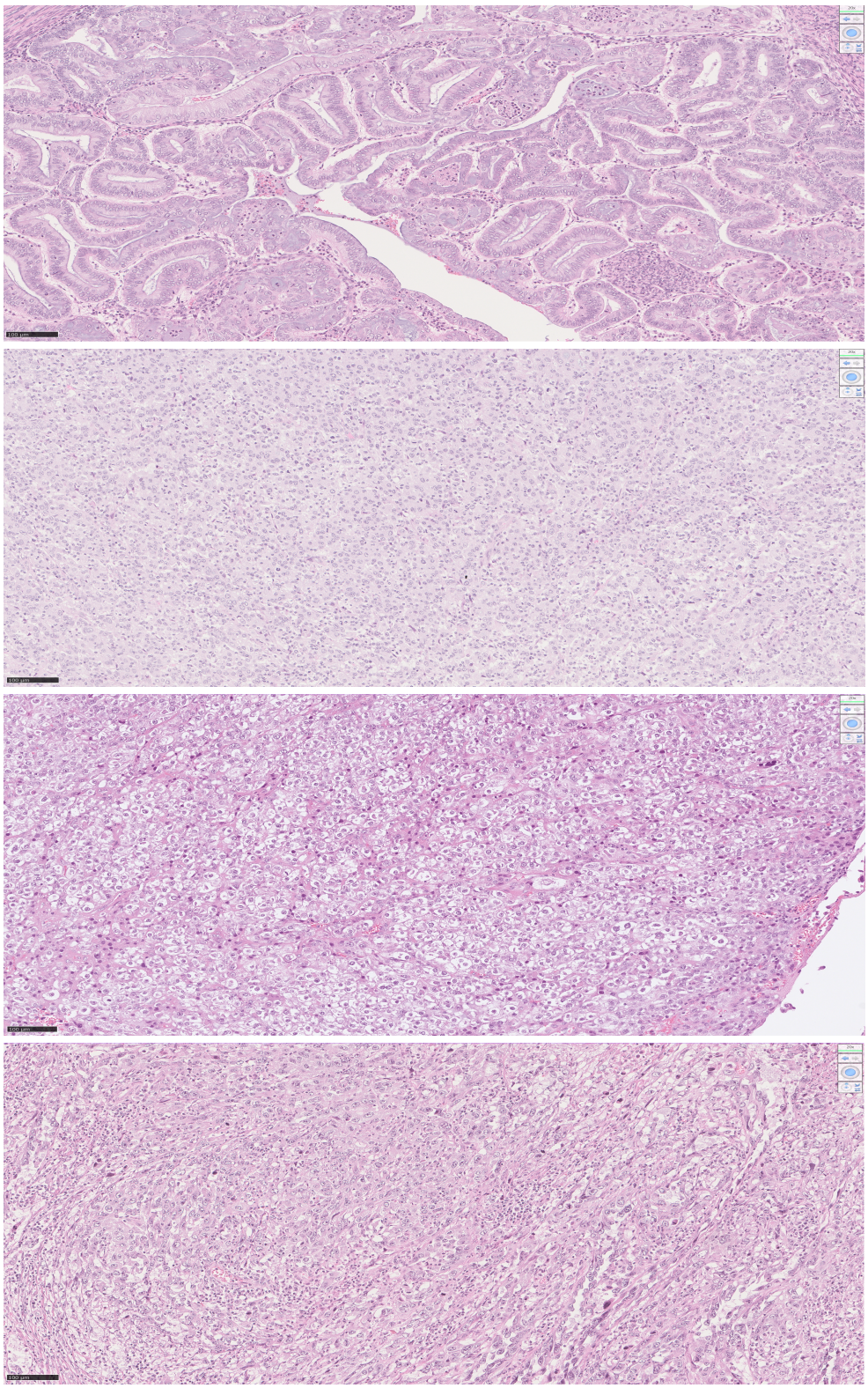

Figure 1. Examples of EC histology. Top two pictures are of endometrioid carcinomas, the top being a low-grade endometrioid cancer FIGO grade 1 with many gland formations and second from top high-grade (FIGO 3) solid growth with no gland formations.

The bottom two are of a clear cell carcinoma, solid growth with many clear highly atypical cells (3rd from top) and bottom is a serous carcinoma with papillary and solid growth and high grade nuclear atypia with many mitoses.

1.4 IMMUNOHISTOCHEMISTRY OF EC

As described above ECs display a wide variety of cytologic features and growth patterns which makes them diagnostically challenging increasing the risk of misdiagnosis[77]. Because of these challenges throughout the years a large number of studies have been focused on immunohistochemistry (IHC) in order to help combat these diagnostic issues. While IHC has been and is a cornerstone of EC diagnostics, the following summating statements in regards to the use IHC in EC diagnostics remain true;

1. There is no magic single IHC marker which will make challenging cases easy to diagnose, therefore the use of IHC panels is advised[78,79].
2. Identifying cases where IHC is necessary can be difficult and determining whether IHC is needed for all cases ("reflex testing") is equally difficult.
3. Interpertation of results dicrepant from both histomorphologic assessment and discrepant from each other in a panel (that is different IHC markers pointing towards different diagnoses in a multiple marker IHC panel) in no easy task.

That said, nowadays it is hard to imagine diagnosing ECs without the use of IHC as they greatly aid diagnosis. Probably the most broadly and frequently used IHC panel consists of p53, Estrogen receptor (ER) and progesterone receptor (PR). This panel is most often used when it is morphologically difficult to distinguish between a low grade endometrioid tumor and a high grade serous carcinoma. This is because most low grade endometrioid ECs are ER+ and PR+ while p53 is seen with a wildtype staining pattern and conversely serous carcinomas are ER-, PR- while p53 stains with a mutation pattern (either strong and complete or complete lack of expression, so called "null" mutational staining pattern)[80]. In fact this simple three stain panel is quite useful in distinguishing high- from low grade tumors as most high grade (serous, clear cell, undifferentiated) tumors are ER negative[81,82]. In the differential between serous and enometrioid carcinomas it is important to remember that roughly 30% of high-grade endometrioid ECs also show a p53 mutated staining pattern[83]. In the same differential another useful stain is p16, as most serous carcinomas a strongly positive while endometrioid carcinomas only show focal positivity[84,85]. Yet another use for the p53, ER and PGR panel is help in identifying high grade areas in mixed carcinomas.

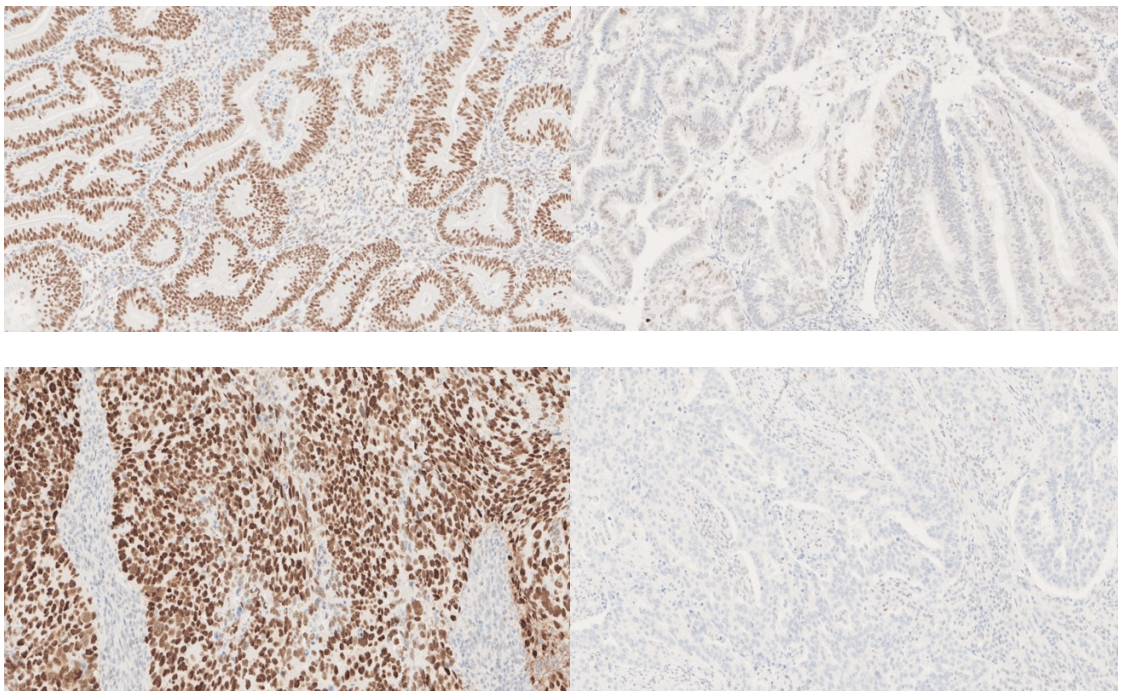

Figure 2. Examples of IHC outcomes. Top left is low-grade endometrioid EC with positive ER. Top right is an example of p53 wildtype-staining pattern with a heterogenous weak positive expression. Bottom pictures are examples of a p53 mutated-staining patterns, on the left a strong homogenous overexpression in all tumor cells and on the right a complete non-expression, so called null-pattern of mutation.

De-/undifferentiated ECs can be diagnostically challenging. These tumors often lose expression of common gynecologic markers ER and PAX8, so identifying them as endometrial primaries can be difficult and a risk of misdiagnosing these tumors as poorly differentiated metastases exists. The difficulties are further compounded when morphologically trying to separate these tumors from solid growing high grade endometrioid and serous ECs. Therefore it was a welcome sight when recent studies showed a large portion of these tumors show aberrant expression/loss of INI1 (SMARCB1), BRG1 (SMARCA4) and BAF250a (ARID1A) [71,86,87], making them useful markers for identifying dedifferentiated ECs. Of note, a small portion of these tumors also showed a p53 mutated staining pattern, perhaps suggesting a different pathogenic (serous carcinoma-like) pathway[86,88,89].

In a small subset of tumors, clinical and radiological checkup cannot reliably differentiate between a primary EC or a primary cervical carcinoma. This is an important distinction because the two cancer types behave and are treated differently. If such a differential arises and morphological distinction alone is too difficult to tell them apart, an IHC panel consisting of CEA, p16, Vimentin, ER can be helpful[90] as Vimentin is much more frequently expressed in ECs and p16 in cervical cancers. One must keep in mind that p16 expression is often seen in high-grade ECs (clear cell, serous carcinoma)[91,92]. If a distinction between the two primaries still remains, an HPV analysis is recommended as cervical cancers are HPV driven/positive. Endometrial biopsies are also sometimes

undertaken in patients with apparent disease in both the ovaries and endometrium at which point a question of primary site arises. If the tumor is serous, which is almost always the case, a WT-1 stain can be useful as it is far more often expressed in ovarian primaries compared to its endometrial counterparts[93,94].

NapsinA and HNF1B have been identified as useful tools in diagnosing clear cell carcinomas of the ovary[95–97]. However, when applying these markers in the setting of EC their usefulness in identifying clear cell carcinoma diminishes. This is due to lack of specificity. While the markers are widely and strongly expressed in endometrial clear cell carcinoma, they are also expressed in a large numbers of serous and endometrioid ECs as well as some benign EC mimics[98].

A large number of other IHC markers have extensively been studied in the setting of ECs. For instance Her-2, Claudins, IMP2 and IMP3 have shown mixed sensitivity and specificity and are of limited clinical use[78,99]. Loss of expression for tumor suppressor PTEN has been shown in endometrioid ECs as well as small subset of undifferentiated and mixed carcinomas[100], but despite efforts to optimize PTEN immunostaining protocols[101] there still remains difficulty in interpreting the PTEN staining results as well as inter-laboratory differences in staining intensity[79], making it a challenging immunomarker to use in everyday clinical practice.

Tumor type	IHC
Endometrioid	ER+, PR+, p53wt, p16 weak and patchy
Clear cell	ER-, p53wt, p16 weak and patchy
Serous	ER-, p53mut, p16 strong
Un-/dedifferentiated	BRG1 and INI1 loss, p53wt, p16 weak

Figure 3. Examples of EC types and their prototypical immunohistochemical phenotype. Carcinosarcoma left out because it is almost exclusively a morphological diagnosis.

1.5 STAGING AND RISK STRATIFICATION

Before 1988, ECs were staged clinically[46,102], but in the late 1980s a series of clinical trials showed discrepancies between clinical stage and risk of extrauterine spread of disease, mostly affecting clinically low grade tumors (about 1/5th of the cases) [102,103]. Since then ECs are staged surgically where a large emphasis of staging has been placed on surgical

pathology to asses myometrial depth invasion (more or less than 50%), presence of lymphovascular invasion (LVSI), involvement of the adnexa (fallopian tube and/or ovaries) and assess for possible invasion of the cervical stroma. The extensive surgical staging includes hysterectomy with bilateral oophorectomy as well as complete pelvic and paraaortal lymphadenectomy. Initially, peritoneal lavage for cytology was also recommended but has since been removed and is no longer considered mandatory for proper surgical staging[104]. However studies have shown that the outcome of peritoneal cytology is an independent prognostic factor[105,106], which is why the latest recommendations suggest considering performing peritoneal lavage in cases with high-grade (especially serous) ECs[107]. The benefits of a comprehensive surgical staging are accurate diagnosis and staging which in turn aids in tailor-making treatment and selection of patients in need of more aggressive adjuvant therapy. In 2009, the FIGO staging[108] classes were slightly modified, but largely remained the same since the late 1980s. They are summarized in *Figure 4.*

Stage I	Tumor confined to the uterus. Ia: Invasion <50% of myometrium. Ib: Invasion >50% of myometrium.
Stage II	Tumors involves cervical stroma. Must invade cervical stroma, tumors confined to endocervical epithelium are not to be regarded as stage II.
Stage III	Tumor outside the uterus confined to the pelvis or retroperitoneum. IIIA: cancer that invades to the serosa or the adnexa. IIIB: Cancer involves the vagina, parametrium or the pelvic peritoneum. IIIC: retroperitoneal node involvement. IIIC1: pelvic node involvement. IIIC2: paraaortic involvement.
Stage IV	Tumor outside the uterus. IVA: invasion to surrounding organs, i.e., invasion of the bowel or bladder. IVB: distant metastasis.

Figure 4. The 2009 FIGO staging of endometrial carcinoma.

In 2016 the ESMO-ESGO-ESTRO[107,109] Consensus Conference led to the formation of a risk group assessment strategy based on the current FIGO staging. The conference recommended a slight revision of surgical staging of ECs with the addition of LVSI assessment and placed a stronger emphasis on histotype determination thus making correct diagnosis even more important. This led to the formation of six risk groups which would aid possible postoperative treatment decisions:

- **Low risk group**: Stage I endometrioid, grade 1–2, <50%myometrial invasion, LVSI negative.
- **Intermediate**: Stage I endometrioid, grade 1–2, ≥50%myometrial invasion, LVSI negative.
- High-intermediate:
  -Stage I endometrioid, grade 3, <50%myometrial invasion, regardless of LVSI status.
  -Stage I endometrioid, grade 1–2, LVSI unequivocally positive, regardless of depth of invasion.
- **High**: Stage I endometrioid, grade 3, ≥50% myometrial invasion, regardless of LVSI status.
  -Stage II
  -Stage III endometrioid, no residual disease
  -Non-endometrioid (serous or clear-cell, undifferentiated carcinoma, or carcinosarcoma)
- **Advanced**: Stage III residual disease and stage IVa.
- **Metastatic**: Stage IVb.

These risk groups, together with a clinical physical assessment of ability to withstand further treatment, lay the base for decisions on postoperative treatment.

Lastly, in this segment on staging, a brief but important note on lymphadenectomy (LA) in ECs. There are no clear definitions on what an adequate LA is and what the extent should be (below or above the inferior mesenteric artery). There are a few studies suggesting a survival benefit for patients whose LAs include at least 10-12 lymph nodes[110–112], and studies suggesting that higher lymph node counts are associated with survival benefits[111], making a pathology based lymph node count a possible surrogate marker of the adequacy of the LA. Furthermore, evidence of patient benefit of LA is uncertain and evidence supporting such practice thin. Large retrospective[113] and single-center studies[111] show overall survival (OS) benefits but large randomized clinical trials[114,115] (RCTs) showed neither OS nor disease specific survival benefits. Therefore, LA should be more considered a staging rather than treatment tool.

1.6 TREATMENT

Treatment decisions are based on the above described risk group assessment[107] which incorporates both tumor stage and grade. While general principles of treatment are generally the same around the world, there is a large variability of treatment selection in any given national guideline. There certainly is a broad international agreement that low grade and stage tumors should be treated conservatively and high grade and stage tumors aggressively. The main uncertainties lie in the intermediate risk groups and with the decision of when a patient is suitable for administration of adjuvant treatment, be it RT or chemotherapy. Nonetheless the general guidelines of treatment for EC are described below.

The mainstay of treatment for all patients with EC is hysterectomy with bilateral oophorectomy. For patients in the low and intermediate ESMO risk groups, LA is not necessary since risk of metastasis is very low[116]. For patients in high risk groups and above, LA for staging is recommended as risk of metastasis is significantly increased (by roughly 5 times)[117].

For the vast majority of EC patients, the above treatments are sufficient for proper staging and even cure with very low risks of recurrence and distant metastases. For patients with stage 3-4 disease (advanced primary and metastatic disease according to ESMO categorization) the focus shifts to radical cytoreductive surgery and if not possible (either because the treatment would be too impairing because of anatomic location or because patients aren't fit enough to endure extensive surgery), multimodal cytoreductive treatment; a combination of surgery and radiotherapy (RT). This has been shown to improve OS and progression free survival (PFS), especially if the cytoreduction is to no gross residual disease[118]. PFS and OS improved from 2,2 to 40,3 and 2,2 to 42,2 months ($p<0.001$) respectively, for patients with cytoreduction to no gross residual disease compared to patients who received no cytoreductive treatment. For advanced disease (bulky stage 3 and 4) cytoreductive surgery should only be considered when there is a chance of no gross residual disease, as surgery for these patients with no hope of clearing all tumor burden does not improve survival rates[119].

Adjuvant radiotherapy, either in the form of vaginal brachytherapy or external beam radiation therapy (EBRT), should be considered for patients in the intermediate and intermediate-high risk groups. While the risk of recurrence is generally low, they benefit from preemptive irradiation to reduce risk of recurrence at the most common local recurrence site[120,121]. Adjuvant radiotherapy is however another hotly contested topic in the EC treatment field, because even though the local risk of recurrence is reduced, adjuvant RT has no impact on OS[120,122], and isolated local vaginal recurrence is curable with isolated RT as sole treatment with success rates up to 100%[123,124]. This is why the current recommendations are only to consider such treatment options and not outright strongly recommend it for all patients.

Local recurrences in the pelvic region, vagina, paraaortal or pelvic lymph nodes, as well as isolated metastatic nodules in the liver or lung, are treated with combination of surgery and RT with relatively good outcomes of 40 % 5-year survival rates[125,126].

Finally, adjuvant chemotherapy (usually paclitaxel and carboplatin combinations) should be considered in stage 1 tumors with non-endometrioid histology as well as in stage 2 tumors with endometrioid histology with adverse factors (deep myometrial invasion, LVSI) as adjuvant chemotherapy reduces risk of local recurrence and metastases[127,128]. In FIGO stage 3 and 4, advanced cancers chemotherapy is recommended as it improves both OS and PFR[129,130].

1.7 PROGNOSIS

Tumor stage is the biggest determinant of outcome and since roughly 75-80% of patients are diagnosed with stage 1 disease the general prognosis for EC is quite good[131]. However, patients with late stage and high-grade tumors have poor outcomes and since most of them present late in life with short expected life expectancies most of them proceed to palliative treatment and best supportive care. The cumulative 5-year survival rates are roughly 80% for all EC patients. Stage associated 5-year survival rates are roughly 95%, 80%, 55% and 20% for stage 1, 2, 3 and 4 respectively[9,131,132].

2 CURRENT ISSUES IN EC DIAGNOSTICS

While some of the issues described below are problematic for us pathologists in everyday practice, it is important to take note of the bigger picture. Incorrect diagnostics lead to a host of problems that are far bigger than any individual diagnosis. While a single misdiagnosis may lead to incorrect under- or overtreatment in that case, a bigger issue lies in problems of standardization of treatment and comparing of clinical trials. It may be that the different results we see in clinical trials are a results of diagnostic discrepancies, making it difficult to contrast and compare outcomes of any given treatment, thus leading to the discrepancies in the way we treat EC patients and sometimes seemingly arbitrary treatment choices that do not affect OS.

2.1 POOR REPRODUCIBILITY

The many overlapping morphologic growth patterns for ECs cause a diagnostic struggle leading to poor inter- and intraobserver reproducibility and thus hardly trustworthy diagnostics. First of all, there is the problem of histotype assessment. In 2013, Gilks et al published a report comparing diagnoses of 3 experienced gynaeceological pathologists in 56 cases of high-grade ECs (serous, clear cell, grade 3 endometrioid and carcinosarcoma). Even with the aid IHC markers, only a 62.5% (35/52) overall agreement with interobserver kappa values ranging from 0.57 to 0.68 (fair to moderate agreement)[133] were achieved. Further studies have substantiated these results showing, at most, moderate agreement in evaluating histotype of ECs[52,53] even when evaluating cohorts of mostly endometrioid ECs.

Second of all, the assessment of percentage non-squamous solid area growth seems at times somewhat arbitrary and difficult but is a key factor in deciding tumor grade[134,135]. Thirdly the diagnostic criteria of nuclear atypia and what constitutes a severe high-grade nuclear atypia were ill-defined at the introduction of the FIGO classification, and assessment of nuclear atypia is and will remain highly subjective[51,55].

To combat these issues attempts were made to simplify the morphologic grading while retaining the grading systems prognostic power (see study number 1 below). Attempts at simplifying the grading by going over to a binary grading system and by assessing more robust features such as tumor necrosis, infiltrative growth pattern and removing the 5% solid growth limit showed slight reproducibility improvements over FIGO (interobserver kappa values 0.55 for FIGO and 0.65 for binary grading)[136]. Binary grading endeavors using architectural pattern, nuclear grade, and mitotic index also showed slight or no substantial improvement over current FIGO system[59,134,137].

Despite these efforts there still remains issues in the histopathological evaluation of tumors with only up to fair interobserver agreement. There is little support suggesting that this is because of poorly defined criteria but rather that the chosen criteria for diagnosing EC are and will remain subjective. This suggests the need to aid morphological diagnostics with

more robust objective tools and perhaps that the purely morphological diagnostic paradigm may have run its course.

2.2 DIAGNOSIS OF ENDOMETRIAL INTRAEPITHELIAL NEOPLASIA

The diagnosis endometrial intraepithelial neoplasia (EIN) is important as it is a precursor to development of EC and the diagnosis can explain uterine bleeding symptoms and guide treatment[138]. There are two main issues with EIN, one is reproducibility of diagnosis and the other is sampling error. There have traditionally been two ways to diagnose these alterations. The WHO94 classification which divided lesions into:

1) simple hyperplasia.

2) complex hyperplasia

3) simple hyperplasia with atypia

4) complex hyperplasia with atypia.

These categories were purely histomorphologically descriptive and clinicians found it difficult deciding course of action based on these diagnoses. There is also ample evidence that this categorization suffered from poor reproducibility between each category with frequent over- and underestimation disagreements[135,139]. However a new classification[140,141] system based their criteria on evaluation of gland to stromal area, cytologic change in focus of altered gland architecture, lesional size of more than 1 mm, and exclusion of cancer and mimics introduced three new categories:

1) benign (benign endometrial hyperplasia)

2) premalignant (endometrial intraepithelial neoplasia)

3) malignant (EC)

This simplified system seems to both increase the pathology reproducibility and simplify clinical decision making for the treating physician while retaining its prognostic power[142,143]. This is why this classification is now the preferred nomenclature for diagnosing premalignant conditions.

The issue with both of these classifications, however, is sampling. Be it by dilation and curettage (D&C) or endometrial suction curette sampling, a large number of concomittant cancers (roughly 40%) are missed when comparing biopsy to final hysterectomy diagnoses in patients with a biopsy diagnosis of EIN or benign hyperplasia[144,145]. Studies suggest that the concomitant use of hysteroscopy during D&C limits the risk of missing coexistent cancers[146,147]. In either case the sampling issue is hard to overcome since technical restraints limit the sampling ability and extent of sampling of the uterine lining. This problem can be mitigated if knowledge that the biopsy finding of EIN or hyperplasia often is associated with EC. Thus patients with an EIN biopsy diagnosis should not simply be

dismissed, but lengthy follow-up and re-biopsies, if not outright hysterectomy, is warranted[148].

2.3 LYMPH NODE MACROSCOPIC EVALUATION AND EMBEDDING STRATEGY

In above paragraphs the surgical issues in regard to incomplete definitions of the extent of LA have been described. While these issues are familiar we also know that lymph node counts can have an effect on both OS and PFR for patients with EC[110,111,149]. However, it is largely unknown what factors affect the number of lymph nodes attained in LAs. Is it just a question of surgical method and technique or does pathological processing have something to do with it? In a study by Euscher et[150] al tissue was embedded from both pelvic and paraaortic LAs in a standard routine way by macroscopically identifying and fractioning lymph nodes, after which the remaining tissue was resubmitted as part of the study to identify to what extent pathologic processing affected lymph node counts. Eusher et al found that the number of lymph nodes did increase but no significantly so (64% of cases had additional lymph nodes after resubmission of tissue), and they found no other additional metastasis after resubmitting more tissue. They did also find significantly more lymph nodes in patients who underwent open surgery instead of laparoscopic or robot-assisted surgery, suggestive the importance of surgical technique.

However, in a more recent retrospective cohort study by Cormier et al[151], they found that the most significant factor in correct staging and lymph node counts was which pathologist was doing the macroscopic evaluation. There clearly is a need for more research in order to precisely define both the surgical extent of LAs , and clear cut macroscopic pathologic procedures and embedding strategies (whole tissue embedding?) in order not to miss any lymph nodes or metastases.

2.4 DISCREPANCY BETWEEN BIOPSY AND HYSTERECTOMY DIAGNOSIS

Since biopsy diagnosis plays such an integral part in surgery planning (pre-operative risk assessment) it is disappointing to note that the diagnosis on biopsy specimen can differ from final hysterectomy diagnosis. This discrepancy may cause the need for additional second look surgery and/or unsuspected adjuvant treatment and is further exacerbated when considering the importance of a correct (low-grade) diagnosis in young women where uterine preserving surgery is a possibility.

There is a large number of studies showing that a significant proportion of patients are upgraded to a higher FIGO grade when comparing biopsy to final hysterectomy diagnosis[114,115,152–154]. The studies show similar results in that between 25-35% of patients are upgraded. Fortunately, the vast majority of these upgrading events are patients being upgraded from FIGO grade 1 to 2. Only 2-5% will be upgraded to high-risk tumors (from FIGO grad 1-2 to grade 3 endometrioid or high risk histotype) resulting in a true clinical impact[155,156].

Again, this is a difficult problem to resolve because most of these discrepancies can be explained by sampling issues. As ECs are heterogenous when the entirety of the tumor can be reviewed it is no surprise that diagnosis can change from a small sample of the tumor. Surely a small subset of these discrepancies can be explained by frank misdiagnosis, especially in cases of villoglandular growth in serous carcinomas, but this problem should be mitigated by the use IHC panels[157,158]. Another important aspect to consider is the prevalence of tumor heterogeneity both in growth patterns and mixed histotype tumors which is quite frequent in ECs[159]. It is important that treating physicians are aware of this grade-shift problem and plan for the possible need of more extensive treatment in cases where patients are upgraded to high-risk.

2.5 P53 IHC INTERPRETATION

Interpretation of p53 IHC can be quite challenging. Since the outcome of such a stain is critical, (positive mutation staining pattern is highly indicative of a high grade, mostly serous, tumor) it is important to be right. While the staining when interpreted correctly is highly congruent with actual TP53 mutational status[160,161], the staining patterns can be difficult to read as 1) it is frequently expressed in normal tissue and 2) can be upregulated/slightly overexpressed due to a number of benign/ reactive causes. In interpreting the status of p53 it is important to follow the rule “all or nothing”. Nonsynonymous missense mutations cause a formidable overexpression in tumor tissue accounting for at least 80% of tumor cells to strongly express p53, or a complete zero-expression (so called null-pattern caused by frame-shift, splice-site or a nonsense mutation) which both should be understood as mutation pattern staining[162,163].

2.6 MYOMETRIAL DEPTH INVASION

Evaluation of myometrial depth invasion is an important prognostic step as it affects tumor stage[47]. However, the thickness of the myometrium varies in different anatomical regions of the uterus which is important to consider when evaluating depth of invasion. Since the stage difference is categorized by invasion of more or less than 50% of the myometrial thickness, the macroscopic evaluation (taking in account where anatomically the piece of tumor is sampled) of myometrial thickness is an important step in surgical pathology evaluation.

A number of studies have reviewed various ways to measure myometrial depth invasion, their prognostic impact and reproducibility. Absolute depth of invasion (DOI, measured in mm from the endometrial myometrium interface to the deepest invasion point) and tumor free distance (TFD, measured in mm from the deepest point of invasion to the uterine serosal surface) are two examples of alternative ways of measuring myometrial invasion. In summary, most of these studies have actually shown that both DOI and TFD can be better predictors of recurrent disease, nodal involvement and disease mortality[164–167], in comparison to traditional myometrial depth of invasion staging practices. However, when studying reproducibility both these variables have been shown to be slightly less reproducible when comparing to traditional myometrial invasion staging practices[168].

Hence, the FIGO staging system has not changed and remains at assessing the 50% mark. I think the most important thing when dealing with depth invasion issues is careful macroscopic evaluation. Fractioning the most deeply invasive part of the cancer clearly and separately so it can be assessed properly and also being careful in measuring depth of invasion in or near fallopian tube entry in the myometrium as the fallopian corner is the point where the myometrium is very thin and depth of invasion can be overestimated.

3 NEW DEVELOPMENTS IN EC DIAGNOSTICS

It should be clear from the past segment that EC diagnostics is in dire need of some fresh and objective methods of diagnostics. There is a rapidly evolving field of genomic studies of EC that in the recent years has greatly expanded our knowledge of EC tumor biology as well as shown great promise of being directly integrated in EC diagnostics and the current morphologic systems of classification.

3.1 EARLY HISTOTYPE SPECIFIC GENE MUTATION PROFILES

As the cost of advanced sequencing has reduced and methods improved and simplified, the field of gene sequencing is now open for possible inclusion into everyday clinical practice. New challenges have however arisen as we learn more about the genetic profiles of different EC histotypes. ECs in general are characterized by high mutational burdens with somewhat overlapping gene profiles (same driver mutations seen in different histotypes) both within and outside of the same histotype. Tumor heterogeneity may further make things difficult as different areas of a tumor may show differing mutational profiles[159,169,170]. For these reasons it is difficult to purely use simple gene profile signatures of a tumor for diagnostics and prognostics without incorporating morphologic and IHC features of a tumor.

Most endometrioid ECs display mutations on PTEN[171] (a tumor suppressor gene), ARID1A[172,173] (chromatin remodeling complex), PIK3CA[174] (Oncogene) and to a lesser extent also KRAS[175] (Oncogene) and CTNNB1[176] (Beta-catenin, Cell adherence regulator and cell division control) mutations. About 1/5th of these tumors also display MSI, DNA aneuploidy and TP53 mutations (mostly in the high grade tumors)[83,177].

Serous tumors show a different profile than endometrioid tumors suggesting molecular screening might be of use in trying to separate these two entities diagnostically. Serous carcinomas instead are almost always TP53 (tumor suppressor gene) and PPP2R1A (cell growth and division gene) mutated. They also very frequently display copy number alteration and DNA aneuploidy[169,172,173,178].

Clear cell carcinomas are hard to clearly define and categorize on a molecular basis because they, as a group, show a very heterogenous molecular profile. A large proportion show a similar profile to that of endometrioid carcinomas with frequent ARID1A and PIK3CA mutations without any other clear-cut separating profile that distinguishes them from their endometrioid counterparts. However, a substantial subset of these tumors show a TP53 and PPP2R1A mutated profile much more akin to serous carcinomas[179–181]. With the novel molecular categorization identified by the TCGA (see more below) it is of note that a fair amount of clear cell carcinomas show a POLE mutated as well as MSI mutational profile.

These tumors tend to biologically and clinically behave as their molecular subgroup irrespective of the histomorhology, meaning that POLEmut clear cell carcinomas display a non-aggressive clinical behavior despite being histologically classified as a high-grade tumors[172,178].

Un-/dedifferentiated carcinomas also display a non-specific molecular profile. They are in part identified with an epithelial to mesenchymal cell-transformation profile (much like carcinomsarcoma) showing downregulation of E-cadherin and upregulation ZEB-1 and HMGA (genes in part responsible for the epithelial-mesenchymal transition)[182]. Roughly 70% of un-/dedifferentiated carcinomas fall into the TCGA categories of MSI and endometrioid-like copy-number low (see below), but are also, to a lesser extent, classified in the other two categories of the TCGA classification[173]. Outside of the TCGA classification these tumors are characterized by a mixed genomic profile with a mixture of endometrioid and serous like profile[183].

As mentioned before, carcinosarcomas are thought to be metaplastic carcinomas (epithelial carcinoma with metaplastic sarcomatous dedifferentiation) rather than true biphasic tumors based on clonal subgroup analysis on both tumor components[184]. Carcinosarcomas as well tend to have either an endometrioid type mutational profile (PTEN and ARID1A mutations) or a serous-like profile (TP53 and PPP2R1a mutations)[172].

To summarize the above, simple targeted sequencing is useful only when specific differentials and questions arise but cannot be used when broader general diagnostic and prognostic questions are considered. While serous and low grade endometrioid tumors have specific mutational profiles, the other remaining histotypes show non-specific profiles that fall within either of the serous or low-grade endometrioid mutational profile categories.

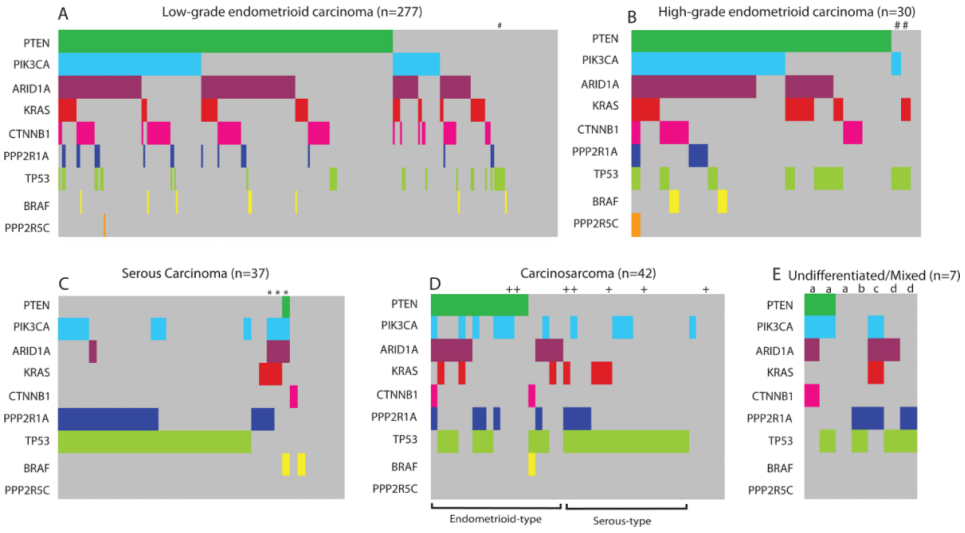

Figure 3. Summary of mutational profiles based on histotype. Colored bars mark mutations and rows mark genes. Republished from reference 172 with permission/license from publisher.

3.2 TUMOR CANCER GENOME ATLAS (TCGA) GENOMIC CLASSIFICATION SYSTEM

In 2013 the TCGA[173] studied 370 ECs and through an integrated system using transcriptomic, genomic and proteomic analyses together with MSI, somatic gene mutations, and copy-number alterations produced a complete genomic based classification system which proved to have prognostic and diagnostic value. The new system described four separate categories of ECs based on complete and extensive integrated genomic profiles instead of single gene mutation profiles described above. The four genomic categories of ECs produced by the TCGA consisted of:

1. POLE-ultramutated

This group is characterized by mutations in the *POLE* gene (exonuclease domain, a subunit of DNA polymerase epsilon), very high mutational burden, frequent mutations in PTEN, KRAS and PIK3CA with slightly less than a third of the cases also showing TP53 mutations. This group is the rarest, roughly 5% of all cases, and are recognized by having very good outcomes with exceptionally low rates of recurrences, distant metastatic disease and death of disease.

2. MSI/Hypermutated

This group is characterized by frequently being of endometrioid histotype, microsatellite instability (MSI) status mostly due to MLH1 promoter methylation, with very high mutation rates, few copy-number variations and often with single gene mutations of KRAS, PTEN, and ARID1A. Hereditary cancers (Lynch syndrome) represent a very small proportion of these cases as most are sporadic and these patients had an intermediary outcome clinical profile.

3. Copy-number low/MSS

Mostly low-grade endometrioid tumors that are microsatellite stable (MSS) with low mutational burden and frequent CTNNB1 and PTEN mutations. Clinical outcomes were intermediary.

4. Copy-number high/”Serous-like”

About ¾ of tumors were of serous histotype and the rest high-grade endometrioid. Characterized by low mutational burden but by frequent copy-number alterations and frequent mutations in TP53 and PPP2R1A. These tumors had by far the worst outcomes.

This original categorization of ECs based on an integrated molecular profile had significant impact on diagnostics, prognostics as well as direct impact on treatment possibilities. It impacted the field of EC research by moving the focus to a more molecular based approach, by itself or as an addendum to classical diagnostics. It shed light on possible targeted

therapies based on molecular profiles. The most important conclusions and impact the TCGA produced can be summarized as:

1. Four genomic categories of ECs each with a distinct set of outcomes. The outcomes were irrespective of histotype and the categorization was an independent prognostic factor.
2. The novel discovery of the POLEmut group has since the TCGA publication independantly proved to have a favourable prognosis with very few recurrances and death of disease[185–188]. This was a critical discovery as a large proportion (roughly 30%) of these tumors have a high-grade endometrioid histology thus suggesting a vast number of EC patients may be overtreated. This discovery is probably the nearest one to make it into clinical care as it suggests that patients that have a "pure" POLEmut profile should receive restrctive treatment regardless of the histotype grade.
3. The identification of highly mutated MSI group which has similar features Lynch syndrome carcinomas with high peritumoral infiltrating lymphocytes (TILs) and growth pattern heterogeneity. This identified a group of tumors with intermediate clincial behaviour and patients who may stand to be most beneficial from receiving adjuvant treatment as well as immunmodulating therapies[189–191].
4. Identification of a TP53 mutated/CN-high group with by far the worst outcomes. This was important in three ways. Firstly a fair number (roughly 35%) of these patients had endometrioid histology with a relatively high number of these actually being low-grade identifying a group of patients where histological underdiagnosis may be relatively common. Secondly, despite the low-grade histology these patients had worse outcomes, this is clinically important as it suggests p53 status on it`s own to be more important indicator of possible need of adjuvant treatment rather than histopathological grade. Thirdly the status of p53mut identifies a patient cohort where aggressive treatment and extensive follow-up is warranted.
5. ECs share some tumor biology and mutational profiles with ovarian cancers, basal like breast cancer and colerectal carcinoma.
6. While many endometrioid and serous carcinomas were genomically different (mostly serous and low-grade endometrioid) many of them shared a genomic profile suggesting the possibility that theses tumors should receive the same type of treatment (p53mut serous and endometrioid tumors).

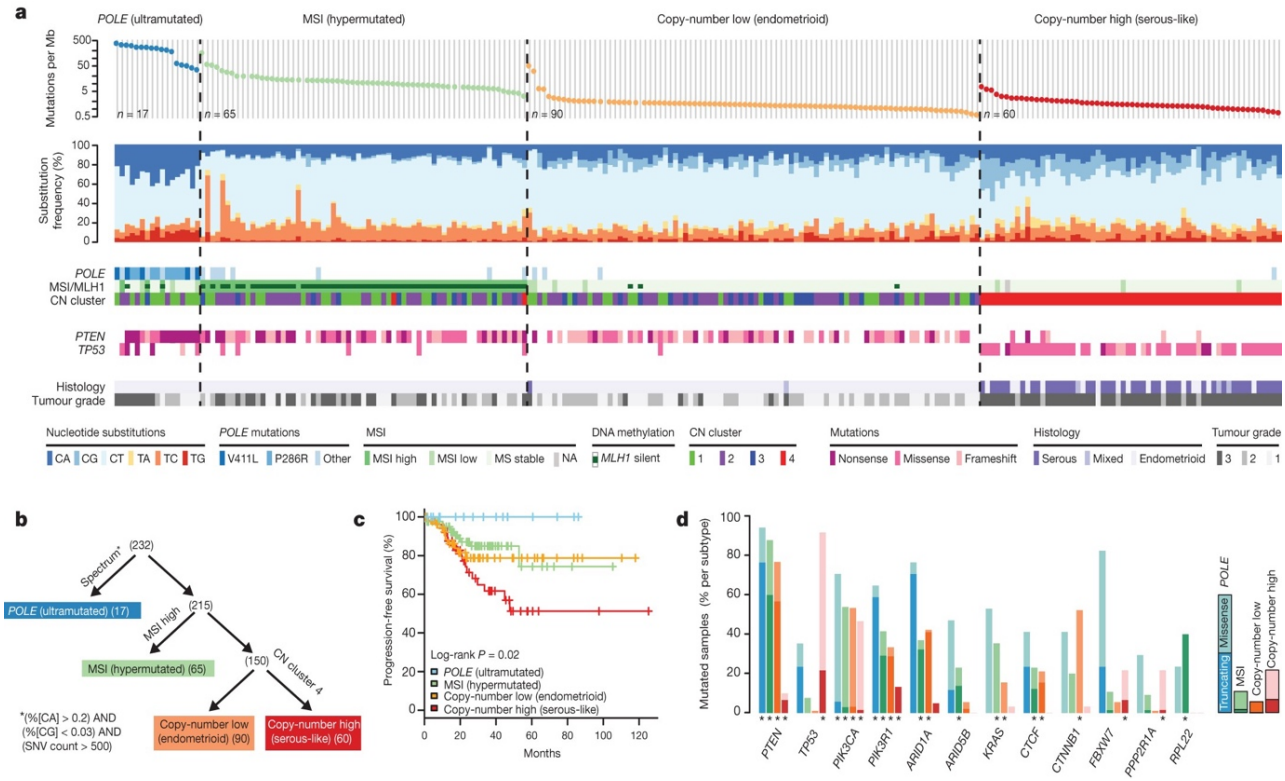

Figure 4. Summary of the TCGA findings. In figure a) a summary of findings for each molecular subgroup in mutations per Megabase (Mb), nucleotide substition frequency, and PTEN and TP53 mutational status. In b) the integrated clustering of cases and number of cases in each subgroup. In c) Kaplan Meier for progression free survival in each molecular subgroup and d) the frequencies of most common gene mutations in each subgroup. Reproduced from reference 173 with license from publisher.

3.3 SURROGATE MARKERS OF THE TCGA CLASSIFICATION

The described methods used by the TCGA are not feasible for use in everyday clinical settings. For one, using fresh frozen tissue with deep sequencing and whole exome analysis was and still is far too expensive and laborious to be used in pathology clinics on a daily basis. Because of this, a number of groups (including ourselves, see papers 3 and 4) have been working on implementing the TCGA findings into the clinics but in a simplified manner using readily available tools that wouldn't cost an inordinate amount nor take excessively long time to complete, to produce so called surrogate TCGA models.

Between the years 2016 and 2018 the Vancouver group published a series of retrospective studies detailing a molecular model they called ProMiseE (Proactive Molecular Risk Classifier for Endometrial Cancer) [192–197]. In entailed trying to replicate the TCGA molecular classifier by targeted sequencing for POLE exonuclease domain mutations (POLEmut group), then using IHC markers for p53 (p53mut or p53wt) and MSI (MSH2, MSH6, MLH1, PMS2 to produce MMRdeficient or MSS) in order to reproduce the four

molecular categories found by the TCGA and see if these surrogate TCGA subgroups remained prognostic. This produced four categories equivalent of the TCGA; MMRd (MMR deficient or MSI), p53wt (equivalent to CN low of the TCGA), p53mut (CN high in the TCGA) and POLEmut (hypermutated/POLEmut in the TCGA). Since the categorization uses simple means and not a complete molecular profile a few issues will arise, firstly there will be cases where are all three molecular steps are neg (MMR intact, p53wt and no POLE mutation), these cases were categorized as p53wt (also called NSMP in other studies, no special molecular profile) and equivalent of the CN-low group in the TCGA. Secondly there will be cases that are positive for more than one molecular marker, so called "multiple-classifiers". In this instance the Vancouver group performed a subgroup analysis of these cases and found the best way to handle them is through an algorithmic approach (see Figure 5 below) where they divide these multiple classifiers into one of the algorithmic groups. This methodology was successful in reproducing the TCGA groups and showed similar predictive properties and produced similar Kaplan-Meier curves as the TCGA classification. The surrogate molecular classifier was an independent prognostic marker of progression free survival (PFS) and the p53mut subgroup was an independent predictive marker of PFS irrespective of histology grade.

In 2015-16 the PORTEC[198–200] clinical trials and NRG/GOG[201] published a series of studies similar to the ProMise methodology described above. The same simplified methodology for classifying ECs in a surrogate molecular model were used as in the ProMiseE reports, however with a slightly different way of designating patients into the four molecular classes. Multiple classifiers were deemed unclassifiable and removed from final analyses, and an additional layer of histopathological factors (LVSI), IHC (L1CAM expression) and molecular analyses (CTNNB1 exon 3 mutation) were used to group patients into four molecular subgroups. These studies showed similar results to ProMisE, a molecular classifier providing independent prognostic information making more information available for targeting patients in need of adjuvant treatment and in a multivariate analysis showing molecular subgroups (p53mut and MSI) to be statistically significant prognostic markers. Both ProMisE and the PORTEC classifier show similar risk discriminatory ability to the ESMO risk stratification system. When clinical and pathological features were integrated with molecular features, they resulted in improved risk stratification.

The surrogate molecular methodology has multiple advantages over both conventional pure morphological diagnostics as well as the TCGA complete molecular classification. The methods used are readily available, paraffin-embedded tissue can be used for analysis, it can be used on biopsies alone as it is concordant with hysterectomy results[195] and therefore yield early prognostic information, the results are reproducible and give an additional layer of objective findings which can be used for prognostic and therapeutic guiding purposes on top of histology and staging.

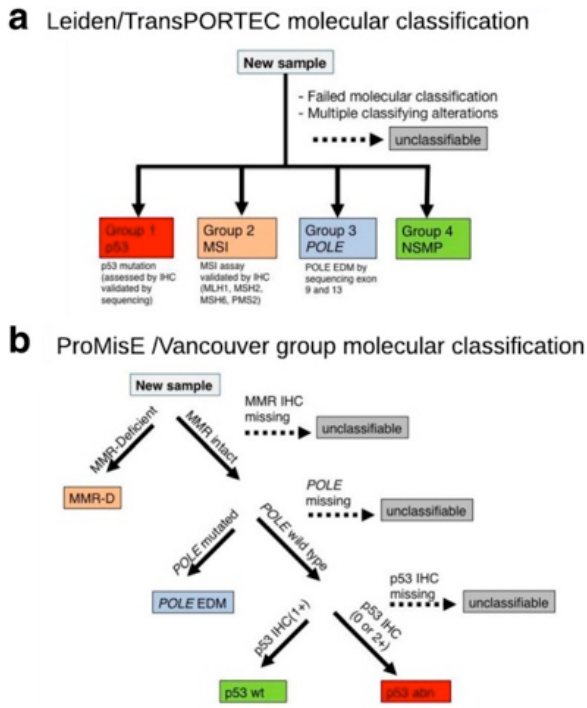

Figure 5. Schematic overview of the methodology used to produce surrogate molecular marker models for both ProMisE and PORTEC. Reproduced from reference 224 with permission from publisher.

3.4 LIMITING FACTORS OF GENOMIC-BASED CLASSIFICATION SYSTEMS

3.4.1 General considerations

A few important things to keep in mind that are limiting factors of genomic models. Firstly, most studies have been performed using a selected high risk patient group in tertiary referral centers with relatively high grade and stage tumors compared to the general garden variety population of ECs. It is therefore unclear whether or not molecular models would hold the same predictive power in non-selected prospective studies. Secondly, it is still unclear how to deal with cases where one or more markers couldn't be evaluated (be it because of poor DNA quality/amount used for sequencing or if one or more IHC markers didn't work). This happens fairly frequently (around 5% in most studies), perhaps these cases should be deemed molecularly unclassifiable. Thirdly, most studies, including the TCGA, have only tested tumors of endometrioid and serous histotypes, posing the question if same conclusions would be derived had unusual types of ECs been included.

In a strict prognostic viewpoint both the PORTEC and ProMisE as well as the TCGA classification systems produce a large intermediary prognostic group consisting of MMR-d/MSI and CN low cases. This is not optimal since the purpose of any predictive/prognostic model is to separate the studied groups as far as possible to create a clearly beneficial and clearly harmful prognostic profile. This large intermediary group could be problematic as

determining treatment strategies can be difficult when outcomes vary so greatly and are intermediate.

3.4.2 MMRd/MSI

MMR proteins correct base-base and deletion mispairing produced during DNA replication in repetitive strands of the DNA called microsatellites. Abruption of the MMR functionality leads to hypermutations in these scattered segments of the DNA[202]. There are many ways to determine a tumor´s MSI/MMR-d status. The most common in the clinical setting for ECs are MSI assays (using PCR for 5-7 known microsatellite foci and comparing tumor to normal tissue coupled with MLH1 methylation analysis) and IHC for MMR-poteins (MSH2, MSH6, PMS2, MLH1). The standardized method of testing in ECs generally follows the Bethesda guidelines for CRC[203]. MMR IHC has shown great concordance with more advanced techniques/assays[30,204,205].

No matter the testing procedure it is important to remember that there are different ways MSI occur, in the TCGA the most common pathway being epigenetic events with MLH1-methylation[173], but there are also somatic and germline (Lynch syndrome associated) specific mutations in each of the affected genes involved in the MMR repair mechanisms. The different ways MSI occur, naturally leads one to think that this should incur different biological, prognostic and treatment specific behaviors in different tumors depending on which way MSI has occurred. However, in both the TCGA and surrogate molecular models all the different types of MSIs are grouped together under the MMRd/MSI-group. This may lead to problems in the future as perhaps a subclassification of the different types of MSIs should improve both prognostics and tailor-making specific treatments.

As an example of the above heterogenous MSI problematics, an NRG/GOG study[206] of a 1000 ECs grouped patients into four categories: normal/MSS, epigenetic defect, probable mutation or MSI-low. Epigenetic and probable mutations were associated with higher grade, stage and LVSI. There were no differences in OS between the groups, however, PFS was better for the epigenetic events group compared to the MSS group but only in a univariate analysis (not after adjusting for age). This study shows the many faces the heterogenous MSI group can have, as the hypermutated phenotype produces complex tumor-lymphatic system interactions and changes tumors susceptibility to chemotherapy. There is no question that the MSI group needs deeper analysis in order to separate both the aggressive behaving and treatment susceptive tumors from within this heterogenous group of tumors.

3.4.3 POLEmut

The POLE gene encodes the catalytic subunit of DNA polymerase epsilon responsible for DNA repair and replication. Mutations of the POLE gene can be diagnosed using Sanger sequencing of exons 9-14 with high accuracy. Mutations lead to a hypermutated phenotype and mutations are most often found in hotspots V411L, P286R and S459F[188,195]. One of the problems in this subset of ECs is answering the question of when a non-hotspot mutation

designates a tumor as POLEmut. In other words which uncommon mutations lead to the TCGA POLEmut phenotype?

This a difficult question to answer because at this time since we don't precisely know which uncommon mutations lead to a TCGA POLEmut phenotype. Because of this, the current consensus is a conservative approach when designating tumors to POLEmut surrogate molecular classifier. In other words, POLE variants should be designated as pathogenic *only* if the mutations is known to cause high mutational burden, high numbers of C→A single nucleotide substitutions and the mutation has previously been described in cancer[207]. Another way to approach this issue in the future may be to add analysis of tumor mutational burden (TMB) by NGS as a supplement to genotyping of POLE mutations[208,209].

3.4.4 Multiple-classifiers

Roughly 3-5% of tumors are positive for more than one surrogate molecular classifier, termed "multiple-classifier" category. As each surrogate classifier is supposed to have it´s own specific prognostic, histologic and molecular profile dictating prognosis and outcome, the question becomes how to categorize or allocate the multiple classifier category.

Leon-Castillo[210] et al recently presented a study investigating perhaps the most interesting group of multiple classifiers, which includes p53mut (p53mut and POLEmut, p53mut and MMRd and triple positive cases with p53mut, POLEmut and MMRd) multiple classifiers. As p53mut tumors by far have the worst outcomes it is most important to categorize this group of tumors correctly. In this study they found that multiple classifiers to a large extent shared the same histomorhological features of "pure" POLEmut and MMRd tumors. Furthermore, a large subset of these multiple classifiers actually only showed a clonal p53mut staining pattern (meaning only a well-defined part of the tumor showed clear p53 mutation staining and not the entire tumor) suggesting that the p53 mutational event was subclonal and probably only secondary to the initial driving mutation (MMRd or POLEmut). Further hierarchical clustering using single nucleotide variant (SNV) type and somatic copy number alterations (SCNAs) showed that p53mut-POLEmut tumors clustered with POLEmut tumors and p53mut-MMRd tumors clustered with MMRd tumors. Also, the clinical outcomes (PFS and OS) were very different for the multiple classiers than that of "pure" p53mut tumors further giving support to the idea that a p53mut is a secondary non-driving event in these tumors. Based on these findings they suggest assigning these multiple classifiers to their "partner" non p53mut molecular class.

While the above mentioned study is comprehensive, it only assigned cases of p53mut partnership. There still remains unanswered questions regarding non p53mut paired multiple classifiers (POLEmut and MMRd). In either case, the grouping of these multiple classifiers needs further clarification with more studies focused on the outcomes and features of different classes of multiple classifiers.

3.5 ADDITIONAL FACTORS / SUBCLASSIFICATION BEYOND THE TCGA

The discussion above on the limitations of a molecular classification should be concluded with this; at the time being molecular categorization is not final nor complete. There is still a lot of work to do in order to deal with the biological and prognostic heterogeneity that exists within in each class.

Perhaps the most symbolic for this is the surrogate molecular class of p53wt/NSMP. This group of tumors is negative for all surrogate molecular markers and deemed equivalent to the CN low TCGA category. Recent studies have revealed ways to further subclassify these tumors and perhaps further branch the molecular classifier. These tumors often (30-50%) show CTNNB1 mutations which in several studies have shown worse outcomes[211,212] within this molecular group suggesting the possible need of at fifth molecular group termed "NSMP-CTNNB1".

L1 cell adhesion molecule (L1CAM) is a molecule member of the immunoglobulin superfamily. It has previously been shown to be an early part carcinogenesis of many cancer types[213]. Expression of L1CAM in ECs is associated with advanced stage, lymph node metastasis and high grade. In low stage cancers expression is associated with lower OS and PFS[192,193,214–216]. Adding L1CAM staining onto the molecular classifier would yield further improved subdivision of the NSMP molecular group.

LVSI is defined as unequivocal tumor cells found in an endothelial lined vascular space found outside the invasive front of the tumor. We have long known that presence of LVSI is a negative indicator, increasing risk of local recurrences and local as well as distant metastasis[217,218]. Fairly recently, substantial or extensive LVSI (defined as multifocal LVSI around the tumor, usually of more than 5 blood vessels) has been shown to be more predictive of worse outcomes[219], this applies to all the molecular subgroups of tumor. These results suggest that substantial LVSI should lead to more extensive adjuvant treatment.

Finally, within the NSMP group SCNAs in chromosome 1q32.1 correlated with worse PFS[220]. Interestingly, the old workhorses ER/PR IHC expression, used in EC diagnostics and prognostics for a long time[97,221], don't seem to add additional prognostic benefit in congregation with a molecular classifier[193].

4 NEW DEVELOPMENTS IN EC CLINICAL CARE AND RESEARCH

4.1 IMPLEMENTING A GENOMICS BASED CLASSIFICATION SYSTEM IN PATIENT CARE

Between the years 2016-2020 a series of reviews[222–227] of the genetics of ECs detailed a few approaches that can be taken to incorporate the findings of the TCGA and the TCGA surrogate molecular markers into clinical practice. This new understanding of genomic classification of ECs have led to a better understanding of the tumor biology and added an objective layer of risk stratification and improved prognostication. The hope is that adding this information to current clinicopathologic risk stratification will aid in further stratifying patient groups and tailor-making specific treatment. However, this remains to be proven in future prospective trials. The group of EC patients that may benefit the most from this integrated diagnostic methodology are patients in the intermediary risk groups, since this is where most unanswered questions lie regarding extent and selection of treatment.

4.1.1 Implementing POLEmut profile in patient care

A large number of studies have now shown the excellent outcomes of the POLEmut group in regards to OS and PFR[187,228–231]. As a substantial portion of these tumors are either high grade, high stage or both (and therefore in most of these studies received adjuvant treatment) one must be cautious in interpreting these excellent outcomes and wonder if POLEmut tumors have good outcomes because they are ultramutated, immunogenic and thus highly susceptible to treatment rather than some inherent beneficial tumor biology. There is, however, considerable data to support an innate benevolent behavior hypothesis. In the PORTEC-1 study POLEmut patients had 100% PFS in a 10 year follow up[124,200] without any adjuvant treatment. POLEmut embryonic stem cells did not prove to be more chemo- or radiation sensitive in patients from the same study[232]. The results of the PORTEC-3 trial with high-risk ECs show excellent outcomes in both treatment arms of POLEmut tumors (RT and CTRT). All of the above support the notion of an innate indolent behavior of POLEmut tumors. Perhaps because they are hypermutated and immunogenic the immune system can defeat the tumor on its own. Therefore, the evidence seems sufficient to start clinical prospective trials including POLE mutation analysis and limiting the adjuvant treatment (and perhaps the extent of primary surgery) in patients demonstrating a pathogenic POLE mutation irrespective of histology grade and surgical stage (up to high-risk patients) so as to avoid unnecessary side effects and overtreatment of these patients.

4.1.2 Implementing p53mut profile into patient care

Much like POLEmut there is now substantial data supporting the poor outcomes of the p53mut group of tumors irrespective of histotype/grade and stage[230,233,234]. This suggests

that in the preoperative setting a p53mut profile on biopsy should warrant an extensive surgical staging including LA, peritoneal lavage as well as possible omental resection. There is however an argument to be made that these patients will in most cases be high stage after surgery and thus more focus should be on postoperative adjuvant treatment instead of extensive primary surgery. Prospective clinical trials will have to examine this question further in the future.

The current evidence of poor outcomes is sufficient to support the notion that these patients should receive an intensified adjuvant treatment course. For patients with an intermediate-high risk profile intensifying treatment from VBT to EBRT[235]. High-risk patients with a p53mut tumor benefit from further adding chemotherapy to RT with improved PFS as a result[230].

4.1.3 Implementing MMRd profile into patient care

There is now a general agreement that MMR testing should be performed for all patients with EC, not least in order to discover possible Lynch syndrome[236]. MMRd tumors are similar to POLEmut tumors, frequently showing endometrioid histology with TILs and high mutational burdens. They, however, differ in prognostic significance though as MMRd tumors are intermediary in outcomes. Because they are intermediary it is difficult to determine appropriate extent of adjuvant treatment. There is scarce evidence on how to adjust treatment recommendation for this patient group.

The results from the PORTEC-2 and 3 studies found that VBT is equally as effective as EBRT in decreasing risk of lymph node recurrences in high-intermediate risk patients without any adverse factors (L1CAM presence, substantial LVSI and/or p53mut) in this patient group. Additionally, for high-risk patients the addition of chemotherapy to EBRT did not add any prognostic benefit[230,237]. These results suggest possible benefits of reduction in treatment for high-risk patients.

4.2 ROLE OF MOLECULAR PROFILES IN ADVANCED OR RECURRENT DISEASE

There are few treatment possibilities for patients with advanced EC. No clinical trials exploring possible treatment options in advanced (distant, FIGO stage 3 and above) EC cases in the context of molecular profiles exist. There are theoretical ideas that immune-checkpoint inhibitors (such as PD-1 blockade) may be of benefit for patients with MMRd and/or POLEmut tumors because these tumors carry a high mutational burden, are immunogenic and histologically often seen with numerous TILs[238–240]. POLEmut tumors, however, show a trend towards good outcomes (not enough few cases so far with advanced disease to make a clear-cut conclusion) with current standard treatment regimens possibly indicating no need for immunotherapies for these patients. However, MMRd tumors with recurrent or advanced disease are a prime candidate for possible immune-checkpoint inhibitors.

The most vital question though remains in patients with p53mut tumors and advanced disease, in part because they have the worst outcomes but also because these tumors tend to present with an advanced disease. There is no current logical reason for these to be candidates for immunotherapy, however targeted therapies may be a solution. Several recent studies have shown serous carcinomas (often p53mut) to overexpress Her2 (ERBB2) in between 20-70% of cases[241–243]. A recent phase II trial showed benefits when adding trastuzumab (monoclonal antibody against the Her2 receptor) to chemotherapy in the form of increased PFS[244]. This may be a potential target for future prospective studies in regards to p53mut tumors.

Another possible option is Poly ADP-ribose polymerase inhibitors (PARPi). These agents work by blocking single-strand repair mechanisms leading to conversion to double-strand breaks which are mainly repaired by homologous recombination (HR) mechanisms, in HR-deficient cells this leads to accumulation of double-strand breaks and causes apoptosis[245]. There are recent studies showing high grade ECs, including p53mut ones, have a sizeable proportion of tumors which are HR-deficient[246,247]. There are current ongoing clinical trials investigating the potential effect on PARPi in the setting of advanced ECs.

4.3 PROSPECTIVE CLINICAL TRIALS

The PORTEC-4a[237,248] clinical study is the first prospective study fully incorporating molecular classifiers as one of the main deciding factors for choice of intervention. It includes high-intermediate risk ECs in order to study effect of selecting patients for suitable adjuvant treatment using a molecular based risk profile. It involves two study arms, the control arm with standard VBT and in the study arm patients selected based on their molecular profile to either observation, VBT or EBRT. The study is ongoing and aims to measure effects on local vaginal recurrence as well as overall PFS and OS.

4.4 SENTINEL NODE BIOPSY

As described earlier LA, has been a standard part of surgical staging of ECs for 30 years since it was shown that roughly 20% of early stage patients had metastatic disease[102]. The risk of metastatic disease, however, is lower in low-grade ECs[249,250] and there is no proven effect on OS when LA[114] is performed, only improved surgical staging. This has led to the belief that LA in patients with low-grade ECs may be overtreated and exposing patients to unnecessary surgical risk with increased morbidity[251,252].

Because of the above facts, a large number of studies have investigated various techniques with differing injection sites and tracers in order to establish a routine sentinel node protocol for EC patients with the hopes of finding a suitable staging method so as to not overtreat while at the same time maintaining high degree of surgical staging accuracy. The current preferred method is injection at four points in the cervix with indocyanine green with near infrared fluorescent imaging detecting roughly 85% sentinel lymph nodes, thus making a complete LA redundant in most low-grade ECs, and has since become an NCCN

guideline[253–255]. The positive rate of sentinel lymph nodes using this method is about 10-15%[254].

While there is no standardized way to process sentinel lymph nodes from a surgical pathology point of view, numerous studies have shown a simple “ultrastaging” protocol (1 level of hematoxylin and eosin followed by 2 unstained levels at 250 micrometers followed by IHC keratin cocktail) proved to be sufficient with no added benefit of more extensive staging protocols[256,257].

5 SUMMARY OF RESEARCH AND PUBLICATIONS

5.1 AIMS

General aims of the thesis were to examine different ways to improve EC diagnostics. To achieve this, we used a three-tiered approach using morphological, IHC and biomarkers and finally molecular/genetic markers as described below.

Specifically:

In **study 1** the aim was to evaluate a cell type independent binary morphological system`s performance in the endometrial biopsy setting and to assess a binary grading system`s weaknesses and strengths in comparison to the traditional FIGO grading system.

In **study 2** the aim was firstly to evaluate the interobserver agreement between 12 consultant gynecologic pathologists in biopsy diagnosis of EC. Secondly to evaluate if a biomarker panel (p53, ER, PR, and DNA ploidy analysis) supplementing morphology would improve the interobserver reproducibility. Third, to test whether ‘‘reflex’’ or ‘‘reflective’’ testing is optimal. Lastly evaluate the role of each biomarker in a statistical model to determine which biomarkers are contributing to upgrading and downgrading.

In **study 3** the aim was to investigate if a simplified clinically applicable surrogate TCGA molecular model could predict outcome of a “general population” of ECs and to compare it to current risk stratification models as well as FIGO grade and stage.

In **study 4** we wanted to analyze the clinical and genotypic characteristics of POLEmut tumors. The aim was to evaluate which POLE mutations could be associated with the TCGA phenotypic prognostic group of Ultramutated/POLEmut tumors.

5.2 MATERIALS AND METHODS

In **study 1** the cohort consisted of 70 women who had a diagnosis of EC (endometrioid, clear cell, mixed or serous) and who had both biopsy and hysterectomy specimens available at Karolinska University Hospital in Solna. The hysterectomy specimen was rereviewed to establish an endpoint diagnosis. The pre-surgery biopsies were independently reviewed by three reviewers using the standard FIGO grading system and then rereviewed a week later using a cell type independent (CTI) binary grading system. After review, all biopsies were assigned a consensus diagnosis should discrepancy occur. Reviews were done blindly and cases were shuffled between reviews. The CTI system uses a point scale assessing growth pattern, nuclear atypia and mitotic index instead of histotype assessment.

Agreement was assessed using percentages and kappa where the strength of agreement by k values were interpreted as follows: 0.00 to 0.20, poor; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, substantial; 0.81 to 1.00, almost perfect.

In **study 2** 12 gynecological pathologists reviewed 70 endometrial biopsy slides, first without any aid from biomarker panel and then after aid of biomarker panel after which results of their diagnoses were compared. One part of the study was to evaluate reflex or reflexive testing (all or only some cases deemed difficult), this was done by allowing a change of diagnosis on all cases (reflex testing) or only after the pathologist "ordered" the biomarker panel (reflective testing).

The tumor grade were binarized (high grade and low grade) for statistical analysis. Up- and downgrading was defined as a diagnosis change from low- to high grade or vice versa. The pairwise interobserver agreement between each of the 12 reviewers was calculated using the frequency of percent agreement and Cohen kappa value. The strength of agreement was assessed the same way as described above in study 1. Simple and multiple generalized linear mixed models with the logit link function where used, raters were considered as a randomized variable and other variables were fixed to assess each biomarkers effect on up- or downgrading. For descriptive statistics, we used the odds ratio. A P-value of <0.05 was considered statistically significant.

In **study 3** a collaborative effort between Karolinska University Hospital and Bern University Hospital defined a cohort (KimBer cohort) of 604 women with unselected general population ECs on which a surrogate molecular marker was applied. The molecular markers applied tried to replicate the TCGA defined molecular categories. In doing so, IHC on tissue microarrays (TMA) consisting of MSH2, MSH6, PMS2, MLH1 (defining an MMRd group) and p53 (defining a p53mut group) was performed and Sanger sequencing of exons 9-14 of the POLE gene (defining a POLEmut group) was conducted. Cases negative for all the markers above were called neg molecular markers (also NSMP in other studies, no special molecular profile) and ECs positive for more than one marker were grouped in a multiple classifier positive group.

Associations between predictive models and clinical factors were analyzed using non-parametric tests. Kruskal-Wallis test was used for continuous variables and Fisher-exact, log-rank and Chi-square for continuous variables. In analyzing the surrogate molecular markers predictive performance of OS, Disease specific survival (DSS) and PFS we used univariable and multivariable (adjusted) analysis with both Kaplan-Meier curves and Cox-proportional regression. To compare the performance of today's standard risk assessments versus the surrogate molecular model we calculated Harrell`s C-index for each model`s discriminatory ability.

In **study 4** we sequenced 604 ECs with Sanger sequencing of POLE gene exons 9-14. When sequencing proved a mutation previously not described as a hotspot mutation, a DNA sample of normal non-tumor myometrium was sequenced to rule out germline mutation. After this the different discovered POLE mutations were classified as known hotspot POLE mutations (P286R, V411L, S297F, A456P and S459F), POLE mutations with a previously described high tumor mutational burden which is consistent with a POLEmut phenotype, and POLE mutations of unknown significance.

Fisher`s exact test and independent T-test were used to compare the POLEmut and non POLEmut groups. Survival analysis was calculated using Kaplan-Meier curves and with log rank test. For assessing risk factors for PFS and DSS, Cox regression analysis of all the different parameters (POLE mutation, histology, age, Grade, FIGO, etc) on outcome were analyzed.

5.3 RESULTS

In **study 1** the results show that the CTI and FIGO grading systems were comparable in accuracy of diagnosis (85.7 and 84.3% with kappa values of 0.71 and 0.69 for FIGO and CTI respectively) with similar sensitivity and specificity in discerning between low- and high-grade diagnosis. The interobserver agreement in the biopsy setting was similar between the two classification systems as well. The same misdiagnosis tendencies appeared to be true for both grading systems, in that overgrading low-grade tumors happened in about $1/5^{th}$ of the cases. The assessment of nuclear atypia was the least reproducible marker in both systems.

In **study 2** we found that the interobserver agreement between the 12 pathologists increased from 75,8%, kappa = 0.52 to 84%, kappa= 0.68 after the use of the biomarker panel. Diagnostic agreement to final hysterectomy diagnosis also increased with use of the panel, going from 83.6% to 88.7% agreement after incorporating the panel in biopsy diagnosis ($p<0.05$). There was no difference in interobserver agreement when using reflective or reflex testing. The two biomarkers p53 IHC and DNA ploidy analysis were the only two markers showing significant effect on up-or downgrading of tumors.

In **study 3** we found that a surrogate molecular model performed well in replicating the TCGA molecular classifier and produced similar survival curves. However, the molecular markers predictive ability was not significantly better than current ESMO risk stratification nor could we find any added prognostic benefit when supplementing ESMO with the molecular markers.

In **study 4** we found 38/599 (6.3%) patients with POLE hotspot mutations. An additional 3 tumors had POLE mutations previously described as having high mutational burden, and another 15 tumors had POLE mutations of unclear significance. The POLEmut phenotype patients were significantly associated with lower age, nulliparity and smoking. No significant histopathological morphologic phenotype was found but most tumors were endometrioid and of low ESMO risk group. When comparing outcomes, only one patient with the POLEmut phenotype had a recurrence (1/38, 2,7%) compared to 16.9% (89/526) in the non POLEmut group. A significant difference in regards to PFS ($p=0.023$) was found between the groups but no differences in regards to OS was found since the one patient with recurrence died of disease. A Cox regression analysis however, did not show any statistically significant difference between the POLEmut and non-POLEmut groups although PFS was close to being significant ($p=0.055$, hazard ratio 0.145, 95% CI 0.02-1.043).

5.4 DISCUSSION, CONCLUSIONS AND FUTURE PROSPECTS

From the mid-80s to the early 2010s the research area of EC and EC diagnostics has been fairly dormant without any major significant changes to diagnostics. But the 2013 TCGA article characterizing the molecular profiles of ECs kicked the research area into a frenzy. This is clearly reflected in the timeline of my thesis; at the beginning in the years 2014-2015 much focus lay on morphologic criteria and how to refine and clearly categorize each component of morphologic diagnostics to somehow reach a clear histologic grade with high interpersonal concordance. This was also our main initial focus, namely new ways of histologically define EC diagnostics. In an attempt to study these changes, we conducted **study nr 1** in which the main conclusion is that the CTI system does not appear to add any significant benefit over standard FIGO grading in the biopsy setting. Using nuclear grade (used in both systems) causes the same lack of reproducibility. For the CTI systems, especially in the biopsy setting, the main problems were because of small amount of tumor material making it difficult to assess mitotic index and overall growth architecture as solid squamous areas were difficult to assess together with small amount of tumor material. In the biopsy setting, where lack of tumor representability is a common problem, it does not appear that CTI adds any great benefit compared to existing grading system.

These are, unfortunately, the same conclusions many other studies examining different ways to improve EC diagnostic histopathology came to. It seems the road of strictly morphologic diagnostics of EC has come to an end, and fresh air in the form of biomarkers and molecular pathology was needed to breathe new life into the field and lead to improvements. Thus, in **study 2** we showed significant benefit of adding a simple biomarker panel in improving interobserver reproducibility. Selective use of the panel is sufficient on morphologically difficult cases as testing on all cases was not found to significantly improve interobserver agreement. The study shows great diagnostic benefits when using a simple biomarker panel, and the use was most beneficial when assessing high grade and mixed carcinomas which are diagnostically challenging.

From morphologic and IHC studies focus shifted to molecular/genomic parameters since many research groups in the area had showed great success in replicating the TCGA findings using simpler methods. We demonstrated the same, shown in **study 3** we could replicate the TCGA findings using a simple surrogate molecular. We could, however, unlike many other research groups, not find any added prognostic benefit of a surrogate molecular marker, neither on its own nor when added as supplemental information on top of today`s risk stratification models. We conclude from this that a surrogate molecular marker isn't a better predictive factor in a study consisting of a general non-selected EC cohort. In other words, the less high-grade and high-stage tumor in a cohort the less power a molecular model has. It may mean we were simply underpowered as recurrences and OS events were quite rare in comparison to other studies reporting different results. Nonetheless, there are clear benefits with the addition of genomic data that are not strictly

and only prognostic, as these can aid in patient selection for adjuvant treatment and perhaps even propose patients compatible with targeted therapies and immunotherapies in advanced and/or recurrent disease.

As the molecular/genomic field has evolved and matured between the years 2015-2020, the focus has slightly shifted to subcategorization within each group of molecular classifiers and on integrating genomic data with histopathologic parameters. As such, we conducted **study nr 4** detailing the phenotype of POLEmut tumors. As one of the only studies conducting a full sequencing of exons 9-14 of the POLE gene we found mostly previously described hotspot mutations but also found substantial mutations with unknown significance. Correctly categorizing POLEmut patients as such is important because it signifies exceedingly good outcomes and suggests these patients could benefit from restrictive treatment. This study shows that, when clearly defined as either hotspot mutations and/or POLE mutations with a known hypermutated phenotype, this patient group of POLEmut had very good outcomes with only 1 recurrence in 38 patients. The Cox regression couldn't show any significant difference between POLEmut and non POLEmut tumors, however we believe again this was because we are dealing with a low risk EC population where both events and POLE mutations are rare, and thus we believe we were underpowered.

In general, the field of EC research has matured and is fast moving towards genomic supplementation aiding and supplementing diagnosis, as well as risk stratification and guiding treatment decisions. I believe molecular and histopathological diagnosis has already answered a great deal for a strata of EC patients; namely the low- grade and stage group with no adverse risk factors (such as a p53mut molecular phenotype), it seems that current treatment is plenty sufficient for these patients as they extremely rarely recur and die of disease.

Future research, I believe, will be focused on answering the following two questions; is there a better way to *further stratify molecular groups* and is there room for further stratification within each molecular group? Case in point, which MMRd tumors behave aggressively and which are mainly indolent? More importantly, do any of them, in advanced and/or recurrent disease, respond to immunotherapy and if so which ones. Other examples in the theme of further subdivision and more subclassification; are there more markers that have yet to be evaluated in order to achieve a better subdivision in the NSMP molecular group so we better can predict NSMP behavior. Will the highly aggressively behaving p53mut group branch onto its own, independent of histology, and be subject to targeted treatments early on in treatment decisions irrespective even of stage?

The other main question that hopefully soon will be answered is whether molecular groups onto themselves can guide early adjuvant treatment decisions. While molecular/genomic incorporation is still young in the EC field there is already sufficient evidence that this may be the case and clinical trials are already ongoing trying to answer these questions. I think one of the most exciting findings is the POLEmut group. While early clinical trials will

definitively answer the question of whether these patients need any adjuvant treatment at all, the superb outcomes of these patients suggest a possibility of even more moderate treatment possibilities, perhaps even omission of surgery and a simple wait and watch program may be in the works for the future. The other focus will lie on the other end of the spectrum, patients with high- grade and stage tumors with adverse molecular features. Which of these patients are susceptive for targeted therapies (and if they are susceptible, which targeted therapies) will be a major focus in the future as outcomes for these patients currently are dreadful.

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